A CASE STUDY ON APPLICATIONS OF DNA-LEVEL POLYMORPHISMS IN TILAPIA GENETIC STUDIES AND STOCK MANAGEMENT

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Introduction

Tilapia has become increasingly important in aquaculture in tropical and subtropical countries, such as the Philippines, Taiwan, Israel, some Sub-Saharan African countries like Cote D'Ivoire, Malawi, Benin Republic, etc. It is much appreciated by consumers, being a good and affordable source of protein. It has been cultured intensively for more than four decades; however its genetic resources have been poorly managed.

Problems in fish management still arise in Tilapia production because of its capacity to over breed. At a young age, these fish are able to reproduce, often leading to the overcrowding of fishponds, thus limiting the growth of individual fish. In an effort to address this problem, monosex populations have been produced generally prepared after three methods. The first of these methods, involved the manual inspection and separation of sexes. A second procedure, which is less labor intensive, involves the use of hormones to induce sex reversal in fry-stage fish. The third method employs the discovery that hybridization of two different species would produce skewed sex ratios, some of which tend to nearly 100% male offspring. These and other efforts which now include modern biotechnology tools to produce superior strains in terms of fast growth, tolerance to adverse environmental conditions, resistance to fish diseases, etc. gave roots to a widespread interest in the study of Tilapia genetics.

The problem and the findings (the story)

Stock identification and assessment in Tilapia has been rapidly improved. Morphological description and morphometric analyses were the first tools used to define tilapiine species (Galman and Avtalion, 1983; Pante et al. 1988). But these techniques are rather arbitrary, and biochemical means (i.e. electrophoresis of expressed isozymes) soon found a more

reliable use in such studies (Macarañas et al. 1986, Galman et al. 1988). Biochemical investigations, however, are still limited in that most of the isozymes are affected by environmental and/or developmental conditions (Galman et al. 1979). DNA-level investigations were thus developed for fish genetic studies. Such approach provides direct investigations of the genetic make-up of several fish species, thus eliminating the effects of extraneous factors. Furthermore, polymorphisms (variant forms) in the DNA are highly numerous as compared to that of isozymes. This equips DNA-level analysis with a lot of genetic markers from which accurate stock identification and assessment can be deduced. Some of these DNA markers are described in this paper giving examples of the application of the use of this type of molecular analysis to tilapia research.

Markers at the DNA level

There are two main types of DNA found in the eukaryotic cell, each undertaking a disparate evolution. These are the genomic or nuclear DNA and the mitochondrial DNA (mtDNA); the former being that which is found in the nucleus and is mostly responsible for directing cellular processes, while the latter is exclusively found in the mitochondria and almost independently dictates the functions in this organelle. Markers in the DNA are observable as restriction fragment length polymorphisms (RFLPs). These are variations in the DNA sequence reflected in different lengths of DNA fragments obtained through endonuclease digestion of the DNA molecule. In mtDNA, the RFLP may be analyzed soon after its digestion with restriction enzymes (REs) at specified conditions. For genomic DNA, resultant fragment lengths, however, would be excessively numerous for practical analysis. Instead, portions/subgroups of the genomic DNA are either hybridized or analysed further. One particularly interesting group of genomic DNA is the *satellite* DNA, so called because it forms a "satellite" peak upon absorbance analysis of the DNA. Satellite DNA has no ascertained functions and is simply hypothesized to take part in binding and stabilizing chromosomal proteins, a structural rather than genetic function. Owing to the lack of function, this DNA derivative is thought to experience low selection pressure thus resulting to high degrees of polymorphism (Turner et al.

1991). One other interesting characteristic is that it is mainly composed of repeated structures present in variable amount (termed as variable number tandem repeats or VNTRs), upon which certain analyses may be based.

There are a number of techniques in molecular biology that may be employed in analyzing DNA-level polymorphism. One of the most basic would be the use of enzymes (REs) isolated from bacteria that cleave the DNA at specific sites. For stock identification and/or assessment using mtDNA, this would usually suffice. Southern blotting, the transfer of electrophoresed DNA to a nitrocellulose film, may be done to produce more permanent records (vs. gels) of the fragment patterns. DNA hybridization may also be done to be able to observe or compare homologous DNA sequences. In this technique, a probe is hybridized to the fragment under a given stringency. Polymerase chain reaction (PCR), the amplification of DNA fragments using primers of known sequences, may be employed for those in which prior knowledge on the DNA length (i.e. the sequence) is available. In the case of Tilapia, wherein neither genomic nor mtDNA has been sequenced, arbitrary primers have been used instead, giving rise to the technique AP-PCR or arbitrarily primed-PCR (Harris et al. 1991). Here there is a random amplification of the DNA and the fragments obtained are sometimes referred to as RAPD or randomly amplified polymorphic DNA. Usually, a combination of molecular biology techniques would yield highly informative data.

Mitochondrial DNA RFLP

For reasons mentioned earlier, a DNA-level analysis of genetic make-up proves advantageous against protein analyses. Between mtDNA and genomic DNA, the former would also have certain advantages over the latter, although such advantages are not strictly empirical. To begin with, mtDNA is smaller, making it easier to handle than genomic DNA. Also, it is highly uniform in size, at least among the vertebrates and the invertebrates (i.e. it is 15-18 kb in fish). Due to the maternal inheritance, sequences within a population (most likely originating from a single maternal individual) are highly conserved, so that it is possible to tell the relatedness of the populations of interest (Ferris

and Berg 1992). On the other hand, its rapid evolution, which is ten times that of genomic DNA, provides a scale for the quantification of divergence. By plotting the divergence against the degree of divergence, it is possible to pinpoint the divergence between two species from a common ancestry. Using mice mtDNA, the calibration scale for divergence was found to be 0.02 per 1 million years (Brown et al. 1979).

RFLP analysis of mtDNA may be done through a four-step methodology. The first step involves the isolation and purification of mtDNA using either CsCl- or density gradient centrifugation. The second and third steps following isolation, is the RE digestion and subsequent agarose gel electrophoresis. When the fragments are obtained, these are then compared and subjected to statistical tests (Ferris and Berg 1992).

Several investigations on species and subspecies variation of Tilapias have been successfully shown to generate RFLPs. Some enzymes are only able to distinguish between tilapia species, but as for *Apa*I, differentiation has been shown down to subspecies level of *O. niloticus* (Seyoum and Kornfield 1992) (Fig. 1).

Genomic DNA RFLPs and Satellite DNA

Methods employed in the analysis of genomic DNA include RE digestion, hybridization and amplification via PCR to reveal polymorphic fragments. Several DNA sequences, however, are more practical amplified randomly and thus AP-PCR is used instead to produce RAPDs as was done simultaneously in three different fish species such as *Barbus terazona, Poecilla reticulata* and *O. niloticus* (Harris et al. 1991). The PCR technique is highly specific and may generate accurate reflections of polymorphism. However, this technique requires rigidly controlled set-ups that, for instance, a slight sample contamination may cause sizeable errors.

The entire genome is too large and excessively polymorphic that genetic comparisons using RE digestion hardly facilitates RFLP analysis - if at all. However, certain noncoding and repetitive segments of the genome known as *satellite* DNA, thought to be only structurally functional, have now found use in genetic analyses. It has the advantage of ease in analysis in that it is rather small (less than 1 kb) and thus enables

even sequence comparisons. Despite the small size, these portions of the genome may contain a high degree of polymorphism. Due to these characteristics, the resolution becomes more defined and detailed studies such as linkage analysis and DNA fingerprinting can be done. Also, amount of DNA required for analysis is rather low thus resulting to a conservation of DNA sample resources, which is crucial in certain instances (e.g. imported germplasm or valuable hatchery genetic resources).

Satellite DNAs have been first observed as extra peaks (- thus the term "satellite") generated upon plotting of DNA content against its buoyant density. Four different satellites are typed according to size; (1) macro-, (2) midi-, (3) micro-; and (4) minisatellites which may be selectively isolated through isopicnic centrifugation, pulse-field electrophoresis, agarose gel electrophoresis, and denaturing gel-electrophoresis, respectively. Of these, the mini-(2-100 bp) and the microsatellites (1-4) are best characterized. Most satellite DNA analysis are thus based on these types.

A general protocol involved in satellite DNA analysis involves basic molecular biology techniques. Initially, isolated genomic DNA is digested with an appropriate RE. Satellite DNA, after gel electrophoresis, is seen as heavily stained bands in the DNA smear. Southern blotting is then done for a more permanent record of the fragment patterns. Heavy staining of satellite DNA is due to the presence of this fragment length in large quantities brought about by the multiple restriction sites individually present within a monomer of the repetitive sequence. Furthermore, the generated fragments may be cloned for further analysis, such as PCR and/or sequencing studies, or labelled to be used as a probe in genotyping unknown stocks, mixed or introgressed populations and offspring from parental stocks. This type of analysis was used in differentiating different tilapia species (Franck et al, 1992). This showed the utility of microsatellite genetic markers as tools in distinguishing tilapia species particularly those that look morphologically similar, e.g. *O. aureus and O. niloticus or O. mossambicus and O. hornorum*. In a recent study, Ahmed et al, 2004 using 20 random 10 or 20-mer primers to assay RAPD polymorphisms among 3 genera of Tilapia In Alexandra, Egypt: *Tilapia zillii, Sarotherodon galilaeus, Oreochromis niloticus* and *Oreochromis niloticus*.

Likewise, Hassanien et al, 2004 found genetic diversity using RAPDs among *O. niloticus* different populations in Egypt (River Nile: Cairo, Assuit and Qena and Delta Lakes: Burilla and Manzalla). From the 230 RAPD bands generated, the population from Qena had the highest degree of polymorphism. It was concluded that this outgroup population from Qena has greater potential for use in tilapia breeding programs. In Netherlands, Rutten et al. (2004 a,b) genetically characterized 4 imported Nile Tilapia strains (*Oreochromis niloticus*) using microsatellite markers and investigated phenotypic correlations between body measurements and fillet weights. This has marketability implications, e.g., in the US tilapia fillet is highly favoured because of its good quality.

Another powerful tool developed for plants, AFLP (amplified fragment length polymorphisms) is now being applied by an Isaeli-US research project on the creation of synthetic strain with cold and low salinity tolerance from crosses of 4 species: *Oreochromis mossambicus, O. niloticus, O. aureus,* red *O. niloticus,* and *S. galilaeus*. Out of the 162 AFLP bands scored from 17 primer combinations, about 25% can differentiate parental species and their F1 progeny (Agresti et al, 2000).

Conclusions

The application of DNA-based genetic analysis in tilapia research and stock development and management is still not fully maximized. The limited research available as cited in this paper were carried out separately and independently. They have revealed the potentials of DNA-level polymorphisms as tools for tilapia genetics and management (Delfin and Omitogun, 200). One of the fundamental concerns of Tilapia genetics is the identification and assessment of available stocks, particularly hybrids from parental species. Steps taken at this particular angle provide insights as to how further studies and management actions may be carried out systematically. Data obtained so far from this type of molecular analysis is able to: (1) provide information regarding the discreteness of stocks; (2) quantify introgression within populations; (3) establish genetic variation or relatedness of different stocks; (4) elucidate evolutionary trends within the Tilapiine genera; and should (5) identify loci controlling production traits (or QTLs: quanntitative

trait loci) and (6) serve as models for studies in other fish systems. Such information is indeed valuable to the overall scientific study of Tilapia and to the management programs for its genetic resources necessary for its farming and breeding and development of superioor strains and breeds through MAS: marker-assisted selection.

Can molecular genetics contribute to African aquaculture sustainability?

Durnham et al (2001) made an exhaustive review on the status of genetics in aquaculture for the new millennium and its would-be positive impact to aquaculture sustainability. In this review he mentioned the advances in fish breeding programmes in several countries using knowledge of breeding and inheritance (Mendelian principles) and the emerging science of molecular genetics as applied to enhancement programmes in fish and invertebrate species such as salmon, trout, carp, catfish, sea-bass, tilapia, oysters, prawns etc. The present study on the applications of DNA markers in tilapia genetics and stock management corroborated Dunham et al's review.

The applications of molecular-based knowledge in tilapia genetics and stock management have been confined to developed and some Asian developing countries. The number of molecular markers such as RFLPs and microsatellites as applied to tilapia domestication and strain evaluation has increased drastically over the last 10 years with recent studies (Lee and Kocher, 1996; Kocher et al. 1998; Agresti et al. 1998; McConnel et al. 1998) leading to establishment of tilapia linkage map in UK and US and collaborative national and international breeding programmes such as:

1. The Philippine- ICLARM (International Center for Living Aquatic Resources Management) Genetically Improved Farmed Tilapia (GIFT)
2. The 'YY'-GMT (genetically modified 'YY' male tilapias) in the Philippines, Vietnam, China, Thailand, Fiji and United States (Mair et al. 1995)
3. Production of 100% all-male tilapias in Israel resulting from crosses of gynogenetic *O. niloticus* females and gynogenetic *O. aureus* males (Shirak et al, 1998).

4. Development of synthetic stocks of Tilapia with better tolerance to higher salinities and lower temperatures by UC-Davies and Israel collaboration

Tilapia, an indigenous African fish is widely cultivated fish in Asia and the Middle East. Machena and Moehl, 2001 in their "*Regional summary of African Aquaculture with emphasis on Sub-Saharan Africa* (SSA) presented a 10-year (1988-1997) trend in aquaculture production in 39 SSA countries. They noted that compared with Asian and Middle East production, aquaculture in Africa is primarily small-scale and rural characterized by one or more small (i.e. 100-500 m^2) ponds with only a mean yield of 500kg/ha/year. They also noted the involvement of family labour, involving all family members estimated at 6 individuals and raising tilapia/ and or catfish (*Clarias or Heterobranchus*) species with some limited carp production, mostly *Cyprinus carpio*. The SSA region's small-scale finfish production estimated at 21, 900 mt ((metric tons) which corresponds to the activities of 1.46 M families representing nearly 9 M individuals involved in family scale aquaculture. They also reported that commercial finfish aquaculture is concentrated in Cote d'Ivoire (*Chrysichthys, Clarias*, Tilapia), Nigeria (*Clarias, Heterobranchus*, tilapia, carp), Zambia (tilapia, carp), Zimbabwe (trout, tilapia), Kenya (trout, tilapia) and South Africa (trout). They estimated that commercial finfish production would be 11,350 mt. Commercial tilapia farms reports yields 10,000 - 15000 kg/ha/year while *Clarias* yields reach 20,000 kg/h/yr. In 1988 tilapia represented 42% of the region's production but the figure dropped to 28% by 1997. Similarly the contribution of catfish dropped from 23% to 16%. This was the trend noted, aquaculture development in SSA is very slow. Constraints contributing to the full realization of the potentials of African aquaculture were many such as: lack of clear government policy, poor government support, weak R and D linkages, inappropriate or inadequate technical support, etc.

Discussion questions

1. What are the strengths and weaknesses of DNA-based markers? Why are they preferred over phenotypic or morphological and biochemical genetic markers?
2. Can the Asian or Middle East example of intensive aquaculture be applied to African aquaculture? Why is African aquaculture production very poor? Can molecular genetics help improve African aquaculture?
3. What is the role of African governments in promoting fish production both capture and culture? How can they encourage farmers to increase their fish production or invest in intensive aquaculture?
4. How can government research institutes and universities help farmers in monitoring the genetic quality of their broodstock? Can those kind of breeding programmes in Asia where the research institutes and universities provide genotyping services to the farmers be copied in Africa?
5. How can international agricultural centres and advanced laboratories help boost aquaculture in developing and other poor countries in Africa?

Acknowledgments

Figures 1 and 2 were reprinted respectively from Aquaculture 102, Seyoum & Kornfield 1992 with permission from Excerpta Medica, Inc. and Genome 35, Frank et al. 1992.

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Related Website Links

http://www.aquafind.ait.ac.th/AQUA/readings/html

http://fao.org/biotech/sector4.asp

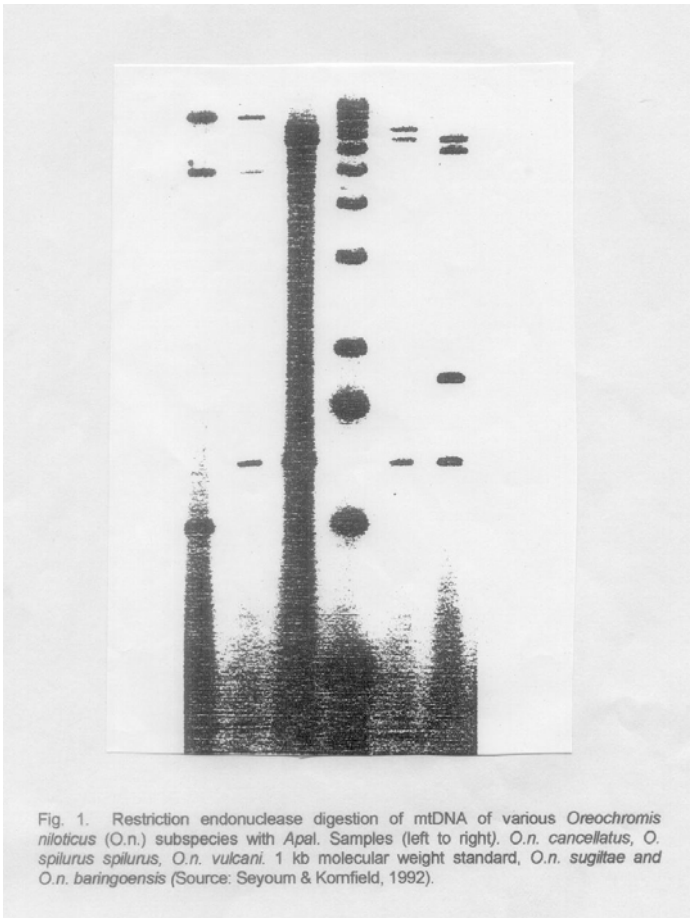
http://iclarm.org/basic research.asp?ct=iclarrm&qu=+%28+GIFT

http://agri-aqua.ait.ac.th?AQUA/readings/YYSEXDET-CJFI.pdf

8. **IMAGES (FIGURES)**

Fig. 1. Restriction endonuclease digestion of mtDNA of various *Oreochromis niloticus* (O.n.) subspecies with *Apa*I. Samples (left to right*). O.n. cancellatus, O. spilurus spilurus, O.n. vulcani*. 1 kb molecular weight standard, *O.n. sugiltae and O.n. baringoensis (*Source: Seyoum & Kornfield, 1992)

Fig. 2. Southern blot and hybridization of a cloned satellite repeat from *O. niloticus* to *Eco*RI digests of various tilapiine DNAs (right panel). Left panel: 2% agarose gel electrophoresis of *Eco*RI digests of genomic DNAs from *O.aureus* (O.aur.), *O. hornorum* (O.horn.), *O.niloticus* (O.nil.), *O. placidus* (O.plac.), *T. zillii* (T. zillii), *T.rendalli* (T.rend.), and the hybrid strain *O. mossambicus x O. hornorum* (O.moss./horn.). Molecular weight markers in base pairs are shown at the left of the figure (Source: Franck et al. 1992).



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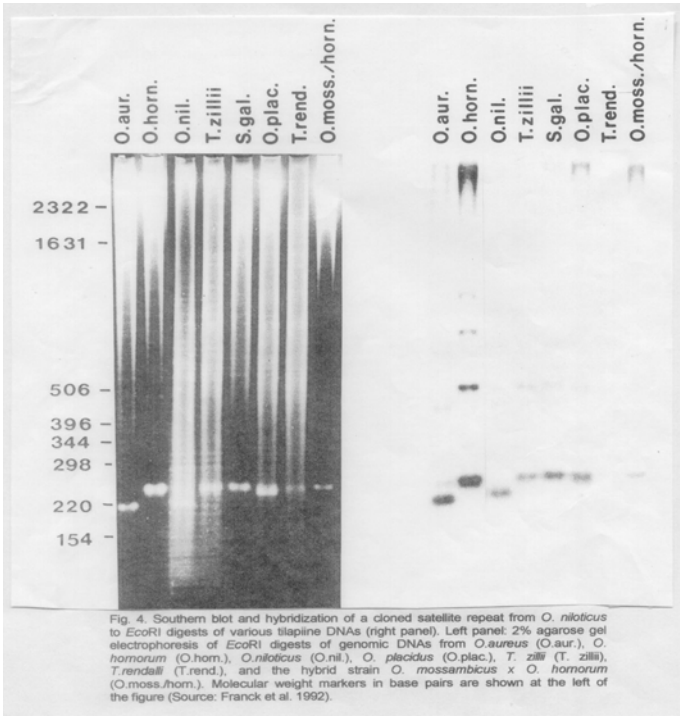

Fig. 4. Southern blot and hybridization of a cloned satellite repeat from *O. niloticus* to *Eco*RI digests of various tilapiine DNAs (right panel). Left panel: 2% agarose gel electrophoresis of *Eco*RI digests of genomic DNAs from *O.aureus* (O.aur.), *O. hornorum* (O.horn.), *O.niloticus* (O.nil.), *O. placidus* (O.plac.), *T. zilli* (T. zillii), *T.rendalli* (T.rend.), and the hybrid strain *O. mossambicus x O. hornorum* (O.moss./horn.). Molecular weight markers in base pairs are shown at the left of the figure (Source: Franck et al. 1992).